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A Monoclonal Antibody against a Synthetic Carboxyl-Terminal Fragment of the Eclosion Hormone of the Silkworm, *Bombyx mori*: Characterization and Application to Immunohistochemistry and Affinity Chromatography

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ABSTRACT—Monoclonal antibodies were produced using as antigen a synthetic fragment corresponding to the C-terminal portion of the eclosion hormone (EH) of the silkworm, *Bombyx mori*. The characterization of these antibodies using ELISA revealed that one of them recognized specifically both the synthetic fragment and native eclosion hormone. Immunohistochemistry using this antibody indicated that EH was produced in two pairs of median neurosecretory cells of the brain. Affinity chromatography of a partially purified EH using a column on which this antibody had been immobilized showed that EH activity was completely adsorbed to this column and eluted with the synthetic fragment with about 60% recovery.

INTRODUCTION

The adult eclosion in the silkworm, *Bombyx mori*, occurs during a specific period of the day [1] as in other lepidopteran insects. The timing of pupal-adult eclosion is controlled by the neurosecretory hormone designated eclosion hormone (EH) [2] which triggers a series of eclosion behaviors. EH acts not only at the adult ecdysis but also at the larval and pupal ecdyses [3]. In *B. mori*, EH might also be involved in the egg hatching behavior [4]. Recently, using the tobacco hornworm, *Manduca sexta*, Copenhaver and Truman have succeeded in identifying a cluster of ipsilaterally projecting cells (group Ia) that contain EH by a sensitive behavioral bioassay and, more specifically, five cells in this group in each brain hemisphere by immunological techniques using an anti-EH antiserum [5].

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We have recently determined the amino acid sequence (61 residues) of the *Bombyx EH* [6]. At almost the same time, Marti *et al.* [7] and Kataoka *et al.* [8] determined the whole amino acid sequence (62 residues) of the *Manduca* EH independently, showing 80% sequence homology with the *Bombyx* EH.

The clarification of the amino acid sequence permitted us to attempt to make monoclonal antibodies against a synthetic peptide fragment. In this paper we describe the characterization of a highly specific monoclonal antibody raised against a synthetic peptide corresponding to the C-terminal portion of EH and its application to immunohistochemical study and affinity chromatography.

MATERIALS AND METHODS

Peptide synthesis

A C-terminal synthetic peptide corresponding to

EH (49-61), H-Cys-Glu-Ser-Phe-Ala-Ser-Ile-Ser-Pro-Phe-Leu-Asn-Lys-OH, was synthesized as follows. The protecting groups for the functional side chains of the amino acids were cyclohexyl ester for glutamic acid, benzyl ether for serine, and 2-chlorobenzyloxycarbonyl (2ClZ) for lysine. Starting with Boc-Lys(2ClZ)-OCH_2-Pam resin, the stepwise solid-phase synthesis was performed on an Applied Biosystems model 430 A peptide synthesizer using dicyclohexyl carbodiimide/1-hydroxybenzotriazole as a coupling reagent. After removal of the protecting groups and resin in HF, the residue was washed several times with diethylether and chloroform by turns. Then the deblocked peptides were extracted with 2 M acetic acid, lyophilized, and finally purified by preparative reverse phase HPLC. Amino acid sequence of the synthetic peptide was checked by an Applied Biosystems model 470 A protein sequencer.

Preparation of antigen

Four mg (0.06 μmol) of bovine serum albumin (BSA) was mixed with 0.3 mg (1 μmol) of N-hydroxysuccinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pharmacia Fine Chemicals) in 0.1 M phosphate-buffered saline (pH 7.5) at 23°C for 30 min, allowing the amino groups of BSA to react with N-hydroxysuccinimide ester moiety of SPDP. After removal of reagents by gel filtration on Sephadex G-25, synthetic EH (49–61) (1.3 mg, 1 μmol) was added to the resulting BSA-SPDP conjugate fraction at 25°C for 20 min. The free sulfhydryl group of cysteine of the synthetic peptide was treated with the 2-pyridyldisulfide moiety of SPDP [9]. After removal of the reagents with Sephadex G-25, about 5.3 mg of EH(49–61)-BSA conjugate were obtained. N-Terminal sequence analysis after purification by HPLC indicated that more than 5 mol of EH(49–61) were coupled with 1 mol of BSA.

Production of monoclonal antibodies

Three female BALB/c mice were immunized four times at a 2-week interval by the intraperitoneal injection of EH(49–61)-BSA (20 μg/mouse) in Freund's complete adjuvant. Three days after each injection, the mice were bled from the tail vein and the blood was centrifuged to remove cells. The antibody detection in each mouse serum was carried out by the dot-immunobinding assay, essentially according to Hawkes *et al.* [10]. After four times of antigen injection, the antibody activity of one mouse detected by the dot immunobinding assay using the synthetic EH(49–61) fragment was positive at 5,000 fold dilution of the antiserum, and this mouse was used to produce monoclonal antibodies.

The splenocytes collected (8×10^7 cells) were fused with mouse myeloma NS-1 cells using polyethyleneglycol. Hybridoma cells obtained were seeded into 96-well microplates (Falcon, 3072) and cultured in the presence of 1×10^4 peritoneal macrophages per well as a feeder layer. The supernatant of each well was primarily screened by dot-immunobinding assay using EH(49–61)-BSA conjugate or EH(49–61) fragment as an antigen. Positive colonies on this assay were again cultured in 24-well plates, and further screened in the same manner, and positive hybridomas were cloned by limit dilution.

The cloned hybridomas secreting anti-EH(49–61) antibody were intraperitoneally injected into a mouse previously injected with 0.5 ml of pristane, and after two weeks, about 5 ml of an ascites fluid was collected. The monoclonal antibody was partially purified from this ascites by ammonium sulfate precipitation at 20–33% saturation, Sephadex G-25 gel filtration, and DEAE-Sepharose CL-6B ion exchange chromatography.

Competitive enzyme-linked immunosorbent assay (competitive ELISA)

Wells of a 96-well ELISA plate (Sumitomo bakelite, MS-3596F) were coated with 50 μl of 5×10^{-10} M EH(49–61)-BSA conjugate in 0.1 M sodium carbonate buffer (pH 9.6) for 2 hr at 25°C. After washing with 50 mM Tris-buffered saline (pH 7.4) (TBS) and blocking with 3% gelatin (Bio Rad), 50 μl of the monoclonal antibody solution corresponding to 1:200,000 dilution of the ascites and 50 μl of serially diluted test materials were added into each well, and the plate was incubated overnight at 4°C.

The plate was washed with TBS containing 0.05% Tween-20 (TTBS), and reincubated with 50 μl of 1:1,000 diluted horseradish peroxidase

(HRP)-linked anti-mouse immunoglobulin goat serum (AMS, Kirkegaard & Perry Laboratories Inc.) at 25°C for 2 hr. Wells were developed with *o*-phenylenediamine as a substrate for HRP. The enzyme reaction was stopped by addition of 50 μl of 2 M H_2SO_4, and the absorbance at 492 nm was measured.

Affinity chromatography

Partially purified monoclonal antibody obtained from ascites was linked to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) by the standard method. About 5 mg of the partially purified antibody was immobilized to the gel (1 g dry weight) and 3.5 ml of affinity gel was obtained. EH was partially purified from 80,000 heads of *B. mori* through the 11 step purification procedure according to the methods as described previously [11]. This partially purified EH in 100 ml of 0.1 M ammonium acetate (pH 8.5) was applied to the affinity column. The column was sufficiently washed with 0.2 M ammonium acetate (pH 8.5) until the absorbance at 280 nm became below 0.005. Adsorbed materials were eluted successively with 0.2 M ammonium acetate containing 100 μM of S-carboxamidomethyl EH(49–61) fragment, 0.25 M sodium carbonate containing 0.5 M NaCl (pH 8.5), and 0.2 M Gly-HCl (pH 2.5).

The active fraction after the affinity chromatography was acidified to pH 2, and directly subjected to a reverse phase HPLC using VP-318 (Senshu Kagaku). Chromatography was performed by applying a linear gradient of acetonitrile (0.5%/min) in the presence of 0.1% trifluoroacetic acid.

Immunohistochemistry

The *Bombyx* brains from freshly ecdysed pupae, 3-, 5-, and 7- day developing adults were dissected out and fixed in Bouin's solution for 4 hr. The specimens were dehydrated with graded ethanol and embedded in paraffin. Serial sections (7 μm thick) were cut on a rotary microtome and affixed on slide glasses. The sections were deparaffinized by xylene, washed with absolute ethanol, soaked in methanol containing 0.03% H_2O_2 for 30 min to inhibit the endogenous peroxidase activity in the tissue, and washed with TBS. The anti-EH(49–61) IgG of 1:500 diluted solution was applied to the rehydrated tissue section after blocking with 3% gelatin, and incubated overnight at 4°C. Subsequently, the extra antibody solution was removed by washing with TTBS. The tissue sections were incubated with the 500-fold diluted solution of HRP-linked AMS for 2 hr, and rinsed again with 50 mM Tris-buffered saline (pH 7.4) (TBS). Then the sections were mounted with 2.2 mM of 4-chloro-1-naphthol in TBS containing 0.03% H_2O_2.

Whole mount staining was performed using *Bombyx* pharate adult brains. The procedure followed essentially the protocol of Bollenbacher *et al.* (personal communication). The brain was fixed with Bouin's solution for 4 hr. After washing with TTBS, the fixed brain was desheathed and exposed to the 500-fold diluted antibody solution containing 2% Triton X-100 overnight at 4°C. After washing with TTBS, the tissue was incubated with 500-fold diluted solution of HRP-linked AMS for 2 hr at room temperature. The tissue was washed with TBS, and incubated with 1.3 mM of diaminobenzidine in TBS containing 0.02% H_2O_2 for 10 min. Then the reaction was terminated by removing the enzyme substrate solution and washed successively with TBS, water, 70% ethanol,95% ethanol, and 100% ethanol. Finally, the tissue was cleared by methylsalicylate overnight and mounted on a slide.

RESULTS

Generation of the monoclonal antibody recognizing EH

During the first screening of the hybridoma colonies using EH(49–61)-BSA conjugate as an antigen, 10 colonies gave positive immunoreaction. On the second screening using EH(49–61) synthetic fragment, however, only one hybridoma colony among them appeared to produce antibody that recognized this fragment. This hybridoma was cloned and the antibody was produced by the intraperitoneal injection of the hybridoma cells into a mouse. The monoclonal antibody obtained from the ascites fluid was purified partially and characterized.

The class of the immunoglobulin produced by

this clone was identified as IgG by its reactivity to the class-specific goat anti-mouse immunoglobulin sera. The characterization of the immunobinding specificity of this antibody was further accomplished by the competitive ELISA using BSA-EH(49–61) conjugate as a coated antigen on a solid phase. The binding of the antibody to the solid phase was inhibited in a dose-dependent manner when BSA-EH(49–61), EH(49–61) fragment, and "highly purified EH" [11] were used as a competitive antigen added to the liquid phase (Fig. 1), but this antibody did not recognize BSA.

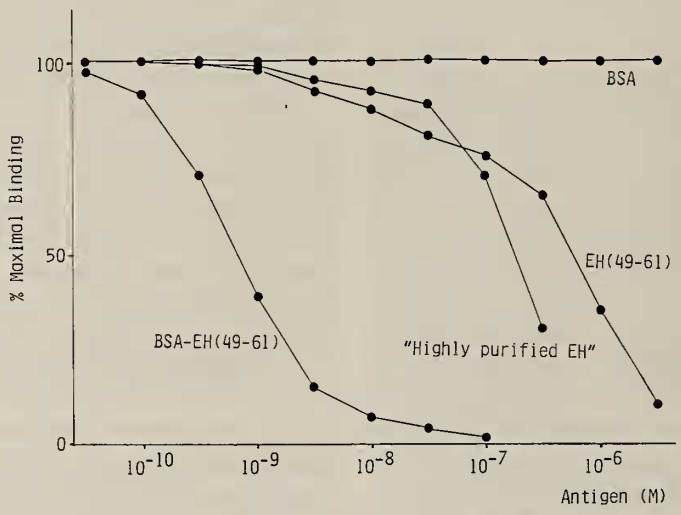

Fig. 1. Binding activity of an EH(49–61) monoclonal antibody as assessed by competitive ELISA to EH(49–61)-BSA. ELISA plate was precoated with 50 μl of 5×10^{-10} M of EH(49–61)-BSA as a competitive antigen and competitive ELISA was performed on this plate with the presence of the monoclonal antibody corresponding to 1 : 200,000 dilution of ascites in liquid phase.

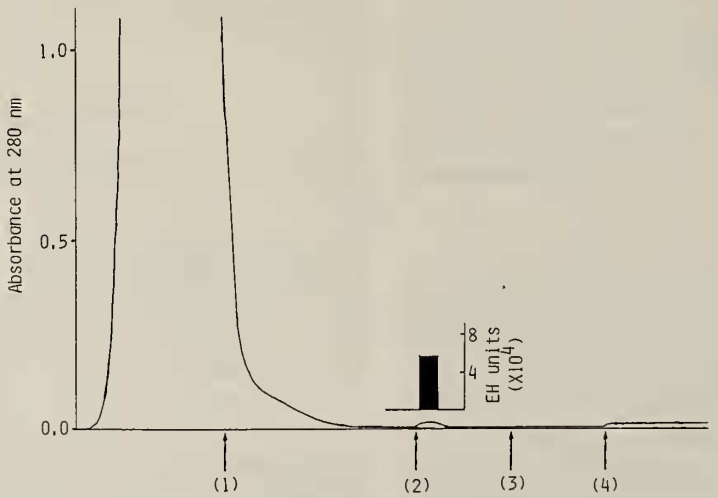

Fig. 2. Affinity chromatography using an EH(49–61) monoclonal antibody-Sepharose 4B column. As a sample solution, 80,000 head equivalents of the partially purified EH preparation was used. After application of the sample, the column was washed with (1) 0.2 M CH_3COONH_4 (pH 8.5), (2) 100 μM S-carboxamidomethyl EH(49–61)/0.2 M CH_3COONH_4 (pH 8.5), (3) 0.5 M NaCl/0.25 M Na_2CO_3 (pH 8.5) and (4) 0.2 M Gly-HCl (pH 2.5) in order. Flow rate was 3 ml/hr and EH activity detected is shown as a solid bar.

Therefore, this antibody not only bound to EH(49–61) portion at a concentration of 10^{-7} M, but also cross-reacted with partially purified native EH. The content of EH in this "highly purified EH" sample was estimated to be about 0.1%.

Affinity chromatography

Partially purified EH was obtained by the usual 11-step purification procedure [11], and this material was applied to the affinity column. Biological activity was completely adsorbed to this column and eluted with 100 μM of synthetic S-carboxamidomethyl EH(49–61) fragment in 0.2 M ammonium acetate buffer (pH 8.5) with about 60% recovery. The column was eluted successively with 0.5 M NaCl containing 0.25 M Na_2CO_3 (pH 8.5) and 0.2 M Gly-HCl (pH 2.5), but EH activity was not detected in either of these two fractions (Fig. 2).

The active fraction was acidified and directly subjected to reverse phase HPLC. Activity was detected in a single peak with a shoulder shown in the painted peak in Figure 3, and Table 1 shows the summary of the purification efficiency. Sequence analysis of the peptide in the main part of the peak for N-terminal portion (up to the 5th residue) assessed the substance in this fraction to be EH.

Immunohistochemical localization of EH in the Bombyx *brain*

Immunohistochemical staining was performed on various stages of *Bombyx* pupal brains. In the usual immunohistochemical procedures using serial sections with 7 μm thick, two pairs of median neurosecretory cells were found to be immunoreactive to the antibody in all stages (Fig. 4A). These cells were about 10 μm in diameter, and their cytoplasm was densely filled with immunoreactive material.

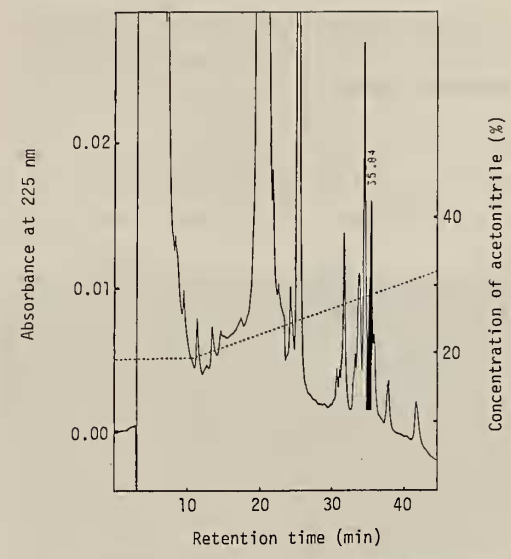

FIG. 3. Reverse phase HPLC of the affinity-purified EH. The result from 10,000 head equivalents of active fraction is shown. The dotted line shows the concentration of acetonitrile in 0.1% trifluoroacetic acid. The shaded peak at 35.84 min indicate fraction containing EH activity and the peak at 22 min is S-carboxamidomethyl EH(49–61).

To assess the topographical distribution of these cells further, whole mount staining was carried out on the brains from the pharate adult 2 days before ecdysis. As shown in Figure 4B, two pairs of median neurosecretory cells were stained, and some immunoreactive nerve fibers were derived from these cells. From various angles of observa-

TABLE 1. Summary of the purification of EH from 80,000 head equivalents of EH sample

Purification step	Weight (μg)	Total activity (EH units)	Specific activity (ng/EH unit)
8th Ppt. with 80% acetone ("Crude EH")	4,400,000	110,000	40,000
9th Sephadex G-50 (fine)	1,960,000	100,000	19,600
11th SP-Sephadex C-25	332,000	90,000	3,700
12th EH(49–61) antibody-Sepharose 4B*	460	55,000	8.4
13th VP-318 (TFA)	4	20,000	0.2

* Activity was eluted with 100 μM S-carboxamidomethyl EH(49–61) synthetic fragment/0.2 M CH_3COONH_4 (pH 8.5).

tion, these four cells were supposed to localize in the anterior portion of the brain as illustrated in Figure 4C and 4D.

DISCUSSION

In our previous study to isolate EH, we obtained only about 30 μg of EH from the extract of 770,000 *Bombyx* pharate adult heads. Because of the limited availability of pure EH, it seemed to be quite difficult to use natural EH for raising anti-EH antibody. Therefore, we took the strategy to make a fragment peptide of EH and to immunize mice with this synthetic peptide after conjugation with BSA. EH has six cysteine residues and these residues were supposed to make three intramolecular disulfide bonds in the molecule to form a globular tertiary structure. Therefore, we decided to make synthetic fragments corresponding to the N-terminal 14 residues and the C-terminal 13 residues, respectively, because these parts would be localized at the surface of the molecule and, therefore, have a rather flexible structure. The study to make monoclonal antibodies against the N-terminal fragment is now in progress.

The attempt to raise the monoclonal antibody against the C-terminal synthetic fragment succeeded in getting one hybridoma clone which secreted an antibody capable of recognizing the native EH as well as EH(49–61) fragment. According to the results from the competitive ELISA, the antibody recognized a partially purified native EH designated "highly purified EH" in a dose-dependent manner. The results showed that our antibody detected the EH molecule in "highly purified EH" at concentrations higher than 10^{-8} M. This assay needs only 50 μl of the sample solution, and so, the amount of EH in 10^{-8} M of the sample solution is about 3 ng. That is to say, this assay system can detect a several hundred fent mole level of EH molecule considering the molecular weight of EH to be about 7,000. This sensitivity is quite satisfactory for immunolo-

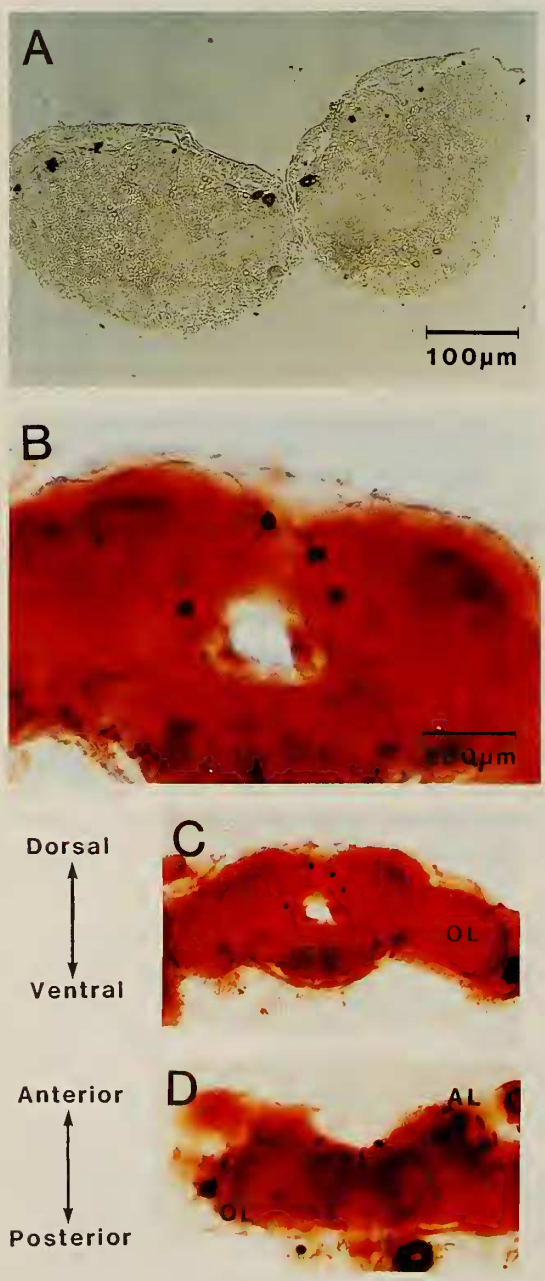

FIG. 4. Immunohistochemical localization of EH in *Bombyx* brain. (A) Transverse section of day-0 pupal brain. In the median part, two pairs of immunoreactive cells are seen. (B, C, D) Whole mount staining of pharate adult brain. (B) Two pairs of neurosecretory cells are stained, and immunoreactive nerve fibers are seen to start from these cells. Anterior view (C) and dorsal view (D) of the pharate adult brain-suboesophageal ganglion complex show that the immunoreactive four cells are supposed to localize in the anterior portion of the brain. AL: antenal lobe. OL: optic lobe. SG: suboesophageal ganglion.

gical assay, but is still less sensitive than biological assay using *Bombyx* pharate pupa, which can detect 0.1 ng of EH.

In our previous study, 18 steps of purification procedure were necessary to isolate EH from the extract of *Bombyx* heads. However, by the use of immunoaffinity chromatography with this antibody, the purification procedure could be simplified considerably by omitting several steps of open column chromatographies and HPLCs.

The EH activity was adsorbed to the affinity column completely. Elution of the active material with the eluant of pH 2.5 did not give a good yield, and the solution containing the synthetic EH(49–61) fragment was concluded to be the best in recovery. Consecutive elution with the solutions of 0.5 M NaCl or of pH 2.5 did not give any active material, indicating that most of the activity was eluted by substitution with the synthetic fragment. In purification by the affinity chromatography using the sample of 80,000 head equivalents, the recovery estimated from total activity was about 60%, and about 440 fold purification could be attained. The specific activity after this affinity chromatography was about 8.4 ng/unit. Considering that specific activity of pure EH is 0.1–0.2 ng/unit, an about 80-fold purification was necessary to isolate EH after this step. By the use of an ODS column, EH was separated from a large amount of S-carboxamidomethyl EH(49–61) and the activity was detected in a single peak. Because the specific activity of this peak was estimated to be 0.2 ng/EH unit and, in addition, the amino acid sequence analysis showed that a peptide in this peak coincided with EH at least for the N-terminal portion, we think EH was isolated by this HPLC step. This immunoaffinity chromatography followed by one-step reverse phase HPLC can considerably simplify the isolation procedure for EH.

Immunohistochemistry using the antibody revealed that two pairs of brain median neurosecretory cells had immunoreactive material in the perikarya (Fig. 4A). Therefore, it is highly possible that these four cells produce EH. Whole mount staining showed that the immunoreactive nerve fibers were originated from these cells as shown in Fig. 4B, and these fibers did not seem to cross at the middle part of the brain. Fugo *et al.* examined the distribution of EH activity in the brain-suboesophageal ganglion (SG) complex of pharate adult *Bombyx* by the surgical cutting into three pieces (median and lateral pieces of brain and SG) and showed that EH activity was highest in the brain median part [12]. Thus, the present immunohistochemical results agree well with this previous report.

Copenhaver *et al.* have already identified EH-producing cells in the moth, *Manduca, sexta* [5]. By an immunohistochemical study using an antiserum against *Manduca* EH, they revealed that 5 pairs of group Ia cells that project to ipsilateral corpus cardiacum and corpus allatum contained EH. It is of interest that there is such a great difference in the number of EH producing cells between the two lepidopteran insects, *Bombyx* and *Manduca.*

Recently, Mizoguchi *et al.* [13] reported four pairs of *Bombyx* median neurosecretory cells contained bombyxin, a *Bombyx* neurosecretory peptide that activates the prothoracic glands of the saturniid moth *Samia cynthia ricini,* by immunohistochemistry using a monoclonal antibody against a synthetic fragment of bombyxin. By a whole mount immuno-staining study using the anti-bombyxin antibody, the bombyxin cells were located in the dorsal-posterior position and proven different from the EH immunoreactive cells (photographs not shown).

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